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GRANT NO: DAMD17-94-J-4463

TITLE: The Role of Basic Fibroblast Growth Factor in Human Breast Cancer

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REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1995	Annual 1 Sep 94 - 31 Aug 95

4. TITLE AND SUBTITLE
The Role of Basic Fibroblast Growth Factor in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4463

6. AUTHOR(S)
Dr. Robert Wieder

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Medicine and Dentistry of New Jersey
Newark, New Jersey 07103

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We found that basic fibroblast growth factor (bFGF), a mitogen, inhibits human breast cancer cell lines. In MCF-7 cells, bFGF binds high-affinity tyrosine kinase FGF receptors (FGFR), activates MAP kinase, induces higher levels of G_1 cyclins D_1 and E and cyclin dependent kinase (cdk) 4, but causes G_1 cycle arrest through induction of cdk inhibitor $p21^{WAF1/CIP1}$. FGF increases association of p21 with complexes of cyclins D_1 and E, inactivates cdk2 and dephosphorylates retinoblastoma protein (Rb). FGF inhibits other mammary cell lines, but only if they do not contain cytoplasmic (18 kD) and nuclear localizing (22 and 24 kD) bFGF. MCF-7 cells engineered to express all three bFGF moieties (MCF-7/NCF) secreted all forms and could not bind exogenous 18 kD bFGF. They were arrested in G_1, had constitutive heterodimerization between FGFR1 and FGFR4, did not phosphorylate MAP kinase, had the same levels of cyclins D_1 and E, cdk4 and $p21^{WAF1/CIP1}$ as controls, did not inactivate cdk2 nor dephosphorylate Rb. They contained inhibitory activity in a cyclin kinase assay and had elevated levels of $p27^{kip1}$. Exogenous bFGF incrementally inhibited MCF-7 cells expressing only 18 kD bFGF, did not increase a constitutive phosphorylation of MAP kinase but raised intracellular $p21^{WAF1/CIP1}$. The data define different roles for different bFGF moieties in breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
basic fibroblast growth factor (bFGF), cyclin D, cyclin E, cdk4, $p21^{WAF1/CIP1}$, FGF receptor, retinoblastoma, $p27^{kip1}$	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

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*U.S.GPO:1993-0-358-779

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TABLE OF CONTENTS

	Page
Front Cover	1
Standard Form 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	8
Conclusions	16
References	17

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THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN HUMAN BREAST CANCER

Annual report, September 28, 1995

INTRODUCTION

The role of bFGF in breast cancer

Among the many gene products which play a role in the complex angiogenic process in breast cancer, basic fibroblast growth factor (bFGF, FGF-2) is one of the most important (1). Cancer cells acquire the capacity to secrete bFGF in a nonclassical manner as one of the last steps to malignant transformation (2). Immunohistochemical stains of primary tumor samples demonstrated that bFGF was not present in hyperplasia or intraductal carcinomas, but was only seen in benign myoepithelial cells or basement membranes surrounding the tumors (3). Once secreted, bFGF is associated with the extracellular matrix (ECM), a complex array of heparan sulfate proteoglycans (HSPG) associated with laminin, fibronectin and collagen (4). bFGF is released from the ECM by collagenases produced by tumor cells and induces migration and proliferation of endothelial cells. bFGF also induces the production of plasminogen activator (PA) in endothelial cells, which further degrades the ECM.

Basic FGF is a ubiquitous member of the heparin binding protein/fibroblast growth factor family of proteins (5). It plays important roles in embryogenesis, muscle development, neural generation and differentiation, angiogenesis and in particular, tumor angiogenesis (6), and normal (7,8) and malignant hematopoiesis (9-12). The protein has extensive inter- and intra-species homology with other members of the heparin binding growth factor family of proteins, of which there are currently nine members identified (13) and some of which are proto-oncogenes, but differs from most of them by lacking a signal peptide which permits classical secretion (14).

The origin and functions of different bFGF moieties

Because bFGF is so ubiquitous and yet has many different specific function in many systems, the specificity of its function and divergence of its signalling potential must necessarily be mediated by many factors at many levels. One mechanism of modulating the activity of bFGF is through the coding of multiple moieties by the same gene, with alternate characteristics. A single mRNA species gives rise to four bFGF moieties using four translation initiation sites. An AUG classical translation initiation site is the start site for an 18 kD species (CIF) and three upstream CUG alternate translation initiation sites result in the synthesis of a 22, 22.5 and 24 kD species (AIF) (15) which have the capacity to localize in the nucleus by virtue of the presence of nuclear-localization sequences (16-18). The cytoplasmic and nuclear species appear to have different roles (19, 20).

Although bFGF does not have a signal peptide, like some other, related members of the FGF family, it is secreted in a non-classical manner by cells which become transformed (2). Nontransformed fibroblast cell lines expressing the 18 kD bFGF moiety proliferate more rapidly, but are not transformed unless a signal peptide is attached to their amino terminal (21, 22). Fibroblasts which express high levels of the 18 as well as the 22 and 24 kD species which localize in the nucleus to high levels (23, Wieder in preparation), are phenotypically transformed. NIH 3T3 cells which express both cytoplasmic and nuclear localizing forms of bFGF only secrete the 18 kD moiety in NIH 3T3 cells (24). Preferential secretion of different moieties is another divergence mechanism.

Basic FGF is mitogenic in many cell types, including fibroblasts, neuronal cells, hematopoietic cells and, as mentioned above, endothelial cells. It was reported that bFGF is slightly mitogenic in MCF-7 breast cancer cell lines in serum-free conditions (25-28), but it inhibited IGF-I induced proliferation of MCF-7 cells in one study (28). In contrast to its effects

on endothelial cells, the 18 kD moiety of bFGF inhibits the proliferation of fibrosarcoma cells (29) and several human breast cancer cell lines (27, 30, 31) when added exogenously in the presence of serum.

We investigated the effects of bFGF in MCF-7, an estrogen dependent cell line (ATCC, Rockvile, MD), and other human breast cancer cell lines. Basic FGF inhibited MCF-7 cell proliferation both in the presence or absence of estradiol. Basic FGF inhibited thymidine incorporation by 50% at 50 pg/ml in MCF-7 cells and reached maximal inhibition at 250 pg/ml. The effect with 500 pg/ml was noticeable after 8 hours and became maximal after 96 hours. Tissue culture kinetics were affected similarly. Basic FGF inhibited the proliferation of MCF-7 cells induced by insulin, estradiol and epidermal growth factor (EGF) and accentuated the antiproliferative effect of TGFβ in these cells. The inhibitory effect by bFGF was reversible by coincubation with neutralizing antibody or by removal of bFGF from the media. Growth inhibition was due to an increase in the G_0/G_1 phase of the cell cycle from 31.7% to 69.9% in estrogen-containing media and from 53.4% to 72.7% in hormone-deprived media ($p < 0.01$). Binding studies and Scatchard curve analyses revealed high affinity binding of bFGF in MCF-7 cells with a constant of 57 pM and the presence of 5,200 sites per cell.

The role of FGF receptors in signal diversity by different members of the FGF family

FGF effects are mediated through binding to one of four high-affinity receptors (32). Basic FGF also binds to cell surface heparan sulfate proteoglycans with low affinity (33), but heparin is necessary for binding to high-affinity receptors (34) and for biological activity (35, 36) through a heparin binding domain of FGF receptors (37) and through interaction with the carboxy terminal of bFGF (38). Heparin exerts its effect by binding to many molecules of FGF, and is responsible for receptor dimerization (39) and can also initiate cellular signalling by receptor activation (40). These molecules, their presence on particular cell types and their preferential interaction to different bFGF's contribute significantly to signal diversity required by bFGF in various systems.

FGF receptors are members of the tyrosine kinase receptor family. Four distinct FGF receptor genes have been identified, which are used by all of the members of the heparin binding FGF family (41). At least two receptors, FGFR1 and FGFR2, give rise to multiple forms due to alternate splicing in the binding domain (42). Different receptors and alternately spliced receptors bind different members of the FGF family with different affinities, giving rise to another layer of diversity in signal specificity (reviewed in 43). The extracellular binding domain consists of three immunoglobulin-like domains which bind the FGFs and induce a conformational change in the receptor. Activated receptors heterodimerize with other FGFR family members and transphosphorylate (44), activating signal pathways unique to each receptor (45). Signalling by FGF occurs partly through cell surface receptors and the cascade they initiate, and partly by transport of the growth factor into the cell (46). Binding of FGF results in nuclear localization through its receptor, in contrast to endogenously expressed bFGF, which remains cytoplasmic (47).

Signal diversity through receptor-initiated pathways

Basic FGF exerts its mitogenic effect by binding to high-affinity tyrosine kinase receptors (48-50) and transiently activating the ERK, extracellular receptor mitogen activated kinase (MAP kinase) pathway (51) through parallel, divergent, convergent and redundant signal pathways which results in the phosphorylation of MAP kinases ERK1 and ERK2. FGFR-4 binds bFGF variably (52-54), has a tissue-specific expression pattern from FGFR1 and 2 (52), causes alternate signalling from FGFR1 (55) and does not cause phosphorylation of MAP kinase (56). Phosphorylation of specific tyrosine moieties of different Src homology (SH2) domains of tyrosine kinase receptors determine the pathway of activation from the receptor (57-59). The receptor-activated signal proceeds through one of several described pathways (60-65).

Induction of the pathways causes rapid transcription of *jun*, *fos* and *myc* (66), causing cells to be released from growth arrest to S phase.

Cell cycle control by cyclins, cyclin dependent kinases (cdk's) and cdk inhibitors

In MCF-7 cells, bFGF activates the ERK pathway and induces tyrosine phosphorylation of $p42^{mapk}$ and $p44^{mapk}$ while inhibiting cellular proliferation, and increases the cellular content of the cyclin dependent kinase (cdk) inhibitor $p21^{WAF1/CIP1}$ (67). p21 exists in a quaternary complex with G_1 cyclins, cdks and PCNA, proliferating cell nuclear antigen (68). The stoichiometric variability of the p21 content of cyclin/kinase complexes determines whether the complex is catalytically active or not (69). PCNA, which activates DNA polymerase δ and is active in excision repair of G_1-accumulated DNA damage, is directly inhibited by p21 with regard to its DNA synthetic function (70) but not to its nucleotide-excision repair function (71). Another mechanism of p21 activity appears to be the inactivation of cyclin/cdk complexes (67), resulting in a decrease in the hyperphosphorylation of retinoblastoma protein Rb (72), which then represses E2F and other transcription factors required for the expression of S phase genes (73).

The wild type tumor suppressor p53, which is induced to high levels by cellular DNA damage, causes cells to arrest in late G_1 by directly inducing transcription of p21 mRNA through binding to *WAF1* upstream sequences (74). Basic FGF can also induce transcription of p21 mRNA using non-p53-mediated pathways in cells with mutant or inactive p53 (75). p53-independent expression of p21 appears to correlate with terminal differentiation (76, 77). The mechanism of induction of p21 by bFGF has not been determined.

Other cyclin kinase inhibitors modulating cell cycle arrest have been discovered. $p27^{kip1}$, a TGFβ-induced cdk4 inhibitor (78) and $p16^{INK4}$ (79) are among the best studied. In addition to proliferation, p16 inhibits Ras-induced transformation (79) and is also associated with differentiation (80).

The phenomenon of dual effects resulting from the stimulation of Ras signalling pathways has precedent in biology, as exemplified by events in embryogenesis (81). Defining how bFGF inhibits some breast cancer cells while stimulating other cell types will contribute to understanding the control of the malignant cell cycle by growth factors.

BODY

Positive and negative signals modulate G_1 cyclins, cdk's and their inhibitors

A. Mitogenic events

Although we found bFGF to be inhibitory in out previous work, it activated the mitogen activated pathway in these cells. Figure 1 shows anti-phosphotyrosine antibody immunoblots of immunoprecipitated ERK2 (MAP kinase) from MCF-7 cells treated with increasing doses of bFGF (ng/ml) for 15 minutes (A) or with 10 ng/ml bFGF for variable times (B). Activation of the pathway is observed by 2 minutes of exposure, but the signal diminishes to low levels by 24 hours, although it remains present. To determine if the inhibitory signal is mediated through the protein kinase C (PKC) part of the pathway, PKC was depleted by a 24 hour incubation with PMA 200 nM or inhibited (nonspecifically) with H7. Significant inhibition of thymidine uptake by these two interventions was further accentuated by addition of 500 pg/ml bFGF, suggesting that the negative bFGF signal transduction is not mediated through PKC.

We studied the effects of bFGF on cyclins, cyclin-dependent kinases and Rb, which govern the passage of cells from the G_1 to the S phase of the cell cycle, in order to understand the effect we were observing. In a bFGF dose-dependent manner in log phase MCF-7 cells, 24 hours after the addition of bFGF there was an increase in cyclin D_1, cdk4 and cyclin E, as determined by Western immunoblots (figure 1C). These initial data demonstrated a bFGF-induced stimulatory signal mediated through the mitogen activated pathway which did not account for the inhibitory phenotype.

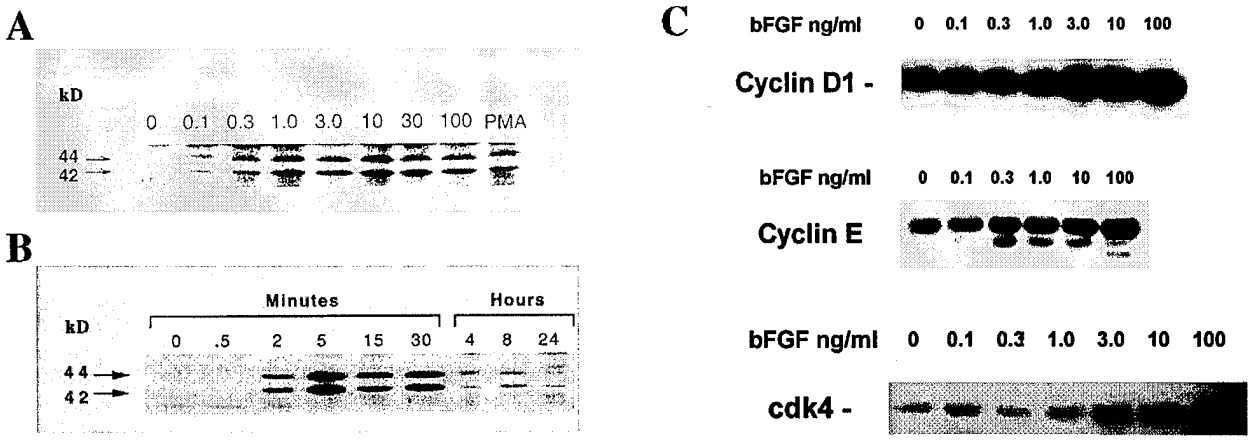

Figure 1. Mitogenic events. Anti-phosphotyrosine immunostained Western blots of anti-ERK2 immuno-precipitates from MCF-7 cells treated with increasing doses of bFGF or with 100 nM PMA for 15 minutes (A) or from cells treated with 10 ng/ml bFGF for varying times (B). C. Western immunoblots with antibodies to cyclins D_1, E and cdk4 of lysates prepared from MCF-7 cells treated with bFGF 10 ng/ml for 24 hours.

B. Inhibitory events

Cyclin A levels were decreased by bFGF, correlating with a decreased S phase cell fraction (figure 2A). Absolute levels of cdk2 were unaffected, but the faster migrating, active form of cdk2 (82) disappeared in a bFGF dose-dependent manner. Retinoblastoma protein converted from its inactive, slower migrating hyperphosphorylated form to its unphosphorylated form upon addition of bFGF in a dose dependent manner. These data were suggestive of the induction of an inhibitor which would account for the phenotypic data.

To assay for an inhibitory effect on catalytic activity, we carried out kinase assays with immunoprecipitated cyclin/cdk complexes from bFGF-treated cells. Immunoprecipitated cyclin A/cdk complexes from bFGF treated MCF-7 cells (10 ng/ml for 24 hours) had less capacity to phosphorylate histone H1 than untreated controls, and lysates were inhibitory when added to cdk/cyclin complexes obtained from Mv1Lu mink epithelial cells (83) as compared to control lysates (figure 2B, + /- bFGF). Control lane shows background histone H1 phosphorylation by unreconstituted Mv1Lu cell lysates, and the cyclin lane shows H1 phosphorylation by cyclin A-reconstituted Mv1Lu cell lysates. The data were similar with cyclin E reconstituted complexes. We found that the cyclin kinase inhibitor $p21^{WAF1/CIP1}$ protein was induced by bFGF in a dose and time (figure 2C) dependent manner by bFGF, as was the $p21^{WAF1/CIP1}$ mRNA (not shown). To determine if p21 induction was a cause and not an effect of G_1 arrest, cells were restricted to G_1 to the same degree by aphidicolin 5 μg/ml for 24 hours as by 10 ng/ml bFGF, but no induction of p21 was evident on Western blot except by bFGF (figure 2D).

To determine if induction of p21 by bFGF was a direct effect, not specific to the G_1 phase, cycle-independent induction of p21 by bFGF was demonstrated (figure 3). Cells were G_1 arrested by aphidicolin for 24 hours, then released and either treated or not treated by bFGF after two hours. Cell cycle distribution and the p21 content of cellular lysates on Western blots were determined every two hours until six hours after bFGF treatment. This experiment suggested that bFGF can induce the accumulation of $p21^{WAF1/CIP1}$ in S phase, demonstrating that p21 induction by bFGF is not specifically a G_1 phenomenon.

The transcription of $p21^{WAF1/CIP1}$ is induced by wild type p53 in many cell types. Preliminary determination by sequencing of PCR products demonstrated that p53 is wild type in this cell line. There was no modulation of p53 levels by 24 hours of bFGF stimulation on Western blots of total cellular lysates.

To determine what effects the induction of cyclin D_1, cdk4, cyclin E and $p21^{WAF1/CIP1}$ along with the decrease in cyclin A, inactivation of cdk2 and dephosphorylation of Rb had on the cyclin/cdk complexes, co-immunoprecipitation experiments were carried out with antibodies to the three cyclins, to cdk 4 and 2, to Rb and to p21. The immunoprecipitated complexes were electrophoresed in an SDS polyacrylamide gel, transblotted to PVDF paper and immunostained with antibodies to cyclin D_1, cdk2, cdk4 and Rb (figure 4). The blots confirmed that there was an absolute increase in total cyclin D_1 and cdk4, and that the total Rb was dephosphorylated and cdk2 was inactivated. There was an increase in the association of cdk4 and cyclin D_1 with the complexes, probably due to the increased levels of cyclin D_1. There was an increase in the association of inactive cdk2 with the complex and in the association of the dephosphorylated form of Rb with the complex. $p21^{WAF1/CIP1}$ association with both cdk4- and cdk2-containing complexes was markedly elevated in cells treated with bFGF. This would account for the inactivation of cdk2 and for the dephosphorylation of pRB due to inactivation of cyclin D/cdk4 complexes and inhibition of histone H1 phosphorylation by cyclins E/ or A/cdk complexes. Co-immunoprecipitation experiments with ^{35}S-labeled cells confirmed these data. These experiments demonstrate that one mechanism of inhibiting cell cycle progression by bFGF in MCF-7 cells is direct induction of $p21^{WAF1/CIP1}$ in a cycle-independent manner, resulting in its increased association with cdk4- and cdk2-containing complexes, inactivation of cdk2 and dephosphorylation of Rb.

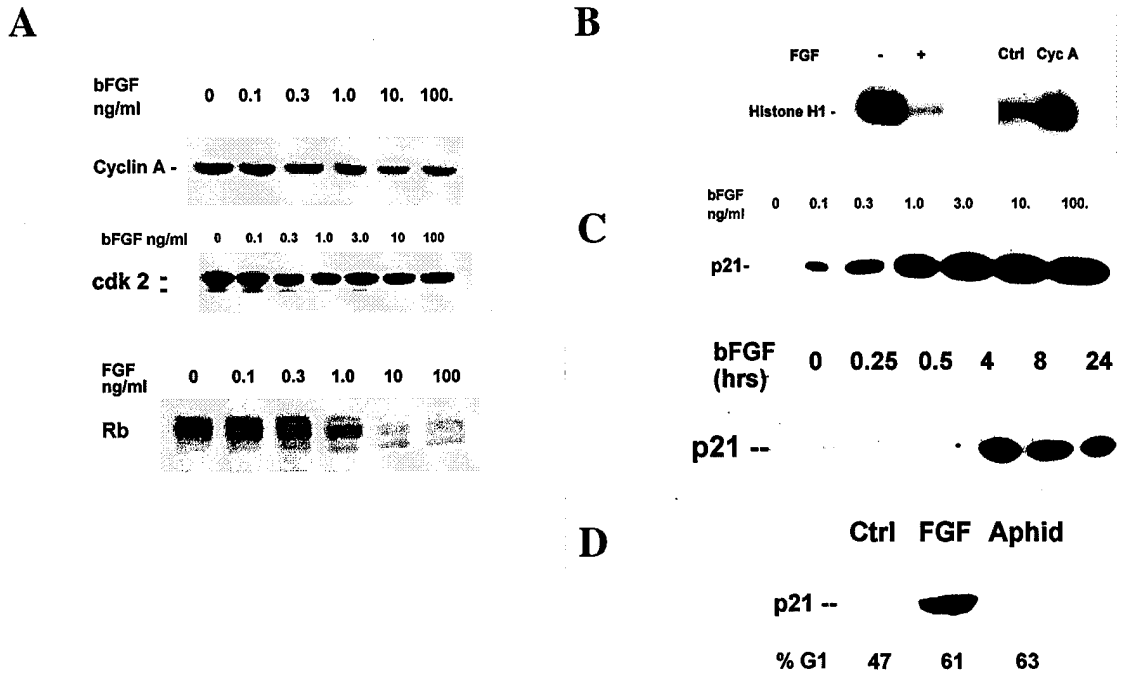

Figure 2. Inhibitory events. A. Western immunoblots with antibodies to cyclin A, cdk2 and Rb of lysates prepared from MCF-7 cells treated with bFGF 10 ng/ml for 24 hours. B. In vitro kinase assay of histone H1 substrate by lysates from MvLu mink lung epithelial cells (Ctr), cells in which cyclin /cdk complexes were reconstituted by addition of exogenous recombinant cyclin A (Cyclin A) and reconstituted mink cyclin complexes to which were added lysates from MCF-7 cells treated with bFGF 10 ng/ml for 24 hours (+) or not treated with bFGF (-). C. Western immunoblots with antibodies to $p21^{WAF1/CIP1}$ of lysates prepared from MCF-7 cells treated with variable doses of bFGF for 24 hours or with bFGF 10 ng/ml for variable times. D. Western immunoblot of lysates from MCF-7 cells treated with media alone (Ctrl) bFGF 10 ng/ml or aphidicolin 5 μg/ml for 24 hours.

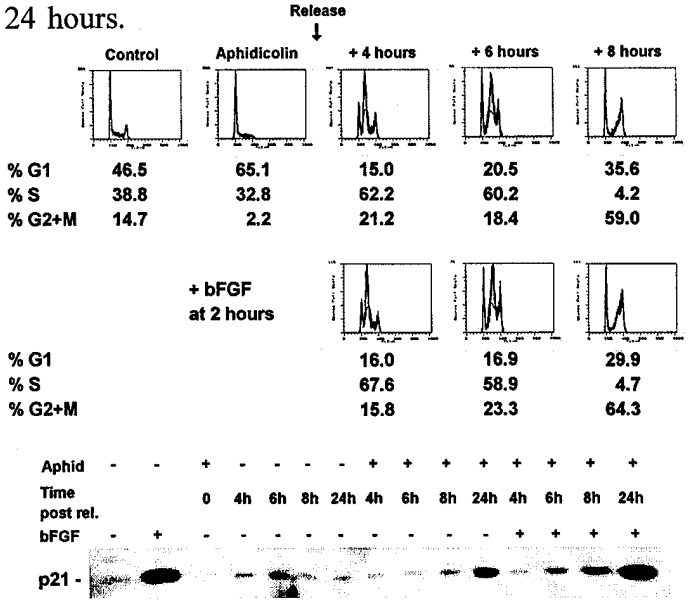

Figure 3. G_1-independent induction of $p21^{WAF1/CIP1}$ by bFGF. Rapidly proliferating MCF-7 cells were cycle arrested in G1 with 5 μg/ml aphidicolin, released for two hours, and incubated with media alone or with bFGF 10 ng/ml for another 22 hours. Cell cycle status was determined every two hours for three measurements after addition of media or bFGF, and simultaneous cell lysates were prepared for Western immunoblots for bFGF. Control cells which were not cycle arrested were also assayed for p21 on Western.

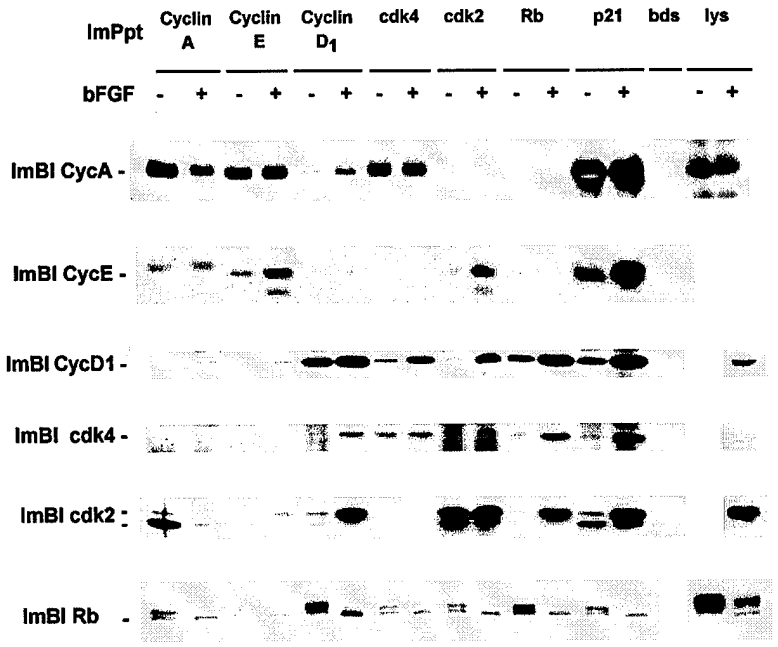

Figure 4. Basic FGF induced-p21$^{WAF1/CIP1}$ associates with G_1 cyclin complexes, inactive cdk2 and unphosphorylated Rb. Coimmunoprecipitation experiments with antibodies to Cyclins A, E, D_1, cdk's 4 and 2, Rb and p21$^{WAF1/CIP1}$ in rapidly growing MCF-7 cells treated with 10 ng/ml bFGF or media alone for 24 hours, immunostained with antibodies to Cyclins A, E, and D_1, cdk's 4 and 2 and Rb (p21 co-migrates with the Ig light chain) and visualized using the ECL system (Amersham).

Basic FGF inhibits other breast cancer cell lines

We found that bFGF inhibited proliferation of other mammary cell lines which did not contain measurable intracellular levels of bFGF, including three MCF-7 lines reported to be stimulated by bFGF in serum-free conditions (25-27) (Table 1; shown are day 7 cells numbers with FGF as a percent of untreated). We also found that cell lines containing 18, 22 and 24 kD moieties of bFGF were not affected by exogenous bFGF (MDA-MB-436 inhibition was marginally significant). These results suggest that our findings are not unique to MCF-7 cells, and that intracellular bFGF levels modulate the response of cells to exogenous bFGF.

Table 1. Response to bFGF in mammary cell lines negatively correlates with bFGF content

Cells	Endog. bFGF	cell# (% ctrl)	p	Cells	Endog. bFGF	cell# (% ctrl.)	p
MCF-7	-	8.3	< 0.0005	MDA-MB-435	+	116.1	> 0.05
MCF-7(L)(30)	-	21.8	< 0.005	MDA-MB-436	+	71.9	< 0.05
MCF-7(P)(29)	-	19.0	< 0.0025	MCF-10	+	129.7	> 0.05
MCF-7(R)(28)	-	55.8	< 0.0025	MCF-12	+	93.0	> 0.05
MDA-MB-134	-	44.2	< 0.005				
MDA-MB-231	-	84.2	> 0.05				
MDA-MB-453	-	32.6	< 0.05				
T47D	-	23.3	< 0.025				

Different moieties of bFGF expressed in MCF-7 cells modulate inhibition through different pathways

The association of a different response of mammary-derived cell lines to exogenous 18 kD bFGF with their intracellular bFGF content suggests an important regulatory role for intracellular bFGF in breast cancer cells. We constructed MCF-7-based cell lines expressing the 18 kD (ΔA), the 18 and 22 kD (ΔS) or the 18,22 and 24 kD (NCF) bFGF moieties using retroviral transduction with a series of N2-based (84) vectors (figure 5A) packaged using the amphotropic cell line G+P-envAm12 (85) as described (86). Cells contained intact vectors on Southern blots, expressed high levels of bFGF by ELISA which were bioactive (87) and expressed the expected bands on Western blot (figure 5B). The 22 and 24 kD moieties in NCF and ΔS localized in the nucleus in fractionation experiments, as predicted (17) (figure 5C). The cells secreted all moieties of bFGF, as shown by 2 M NaCl washes and 10% trichloroacetic acid (TCA) precipitated proteins on a Western blot (figure 6), contradicting observations with NIH 3T3 cells (24). However, NIH 3T3 cells expressing bFGF from the NCF vector preferentially secreted the 18 kD moiety, in agreement with Bikfalvi, et al.'s findings. The secretion of bFGF into the media were measurable after 2 days with a Quantikine ELISA kit, representing a fraction of the secreted bFGF which leached from the surface proteoglycans.

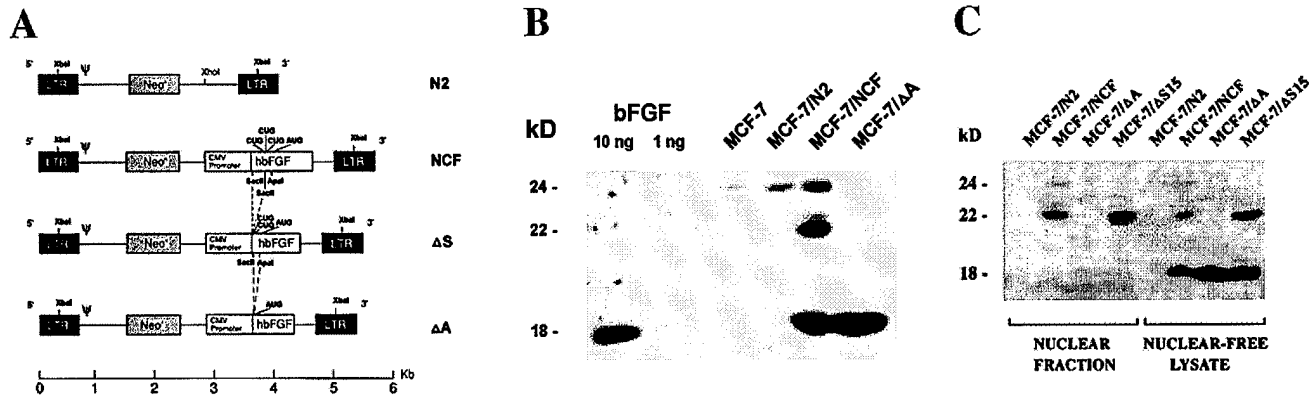

Figure 5. Overexpression and nuclear localization of bFGF in MCF-7 cells. A. N2- based retroviral vectors expressing the 18 kD (ΔA), the 18 and 22 kD (ΔS) or the 18,22 and 24 kD (NCF) bFGF moieties. B. Western immunoblot of lysates from MCF-7 cells containing bFGF vector constructs and of 18 kD recombinant human bFGF using anti-bFGF rabbit IgG antibody. C. Subcellular fractionation of MCF-7 cell constructs depicting nuclear localization of the 22 and 24 kD bFGF moieties.

BASIC FGF CONTENT OF CELLULAR LYSATES AND CONDITIONED MEDIA

	MCF-7/N2	MCF-7/NCF	MCF-7/ΔA
pg bFGF/10^5 cells	1.4 ± 0.1	428.3 ± 8.0	893.1 ± 9.1
pg bFGF/ml c.m.[1] @ 24 hrs	1.4 ± 0.4	2.6 ± 0.2	2.8 ± 0.2
pg bFGF/ml c.m.[1] @ 48 hrs	1.4 ± 0.2	7.2 ± 0.3	9.8 ± 0.1

[1]conditioned media

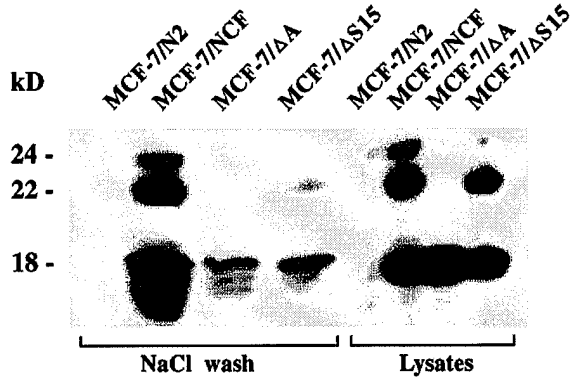

Table 2. Basic FGF content of lysates and conditioned serum-free media after 24 and 48 hours of incubation as measured with a Quantikine Elisa kit (R&D Systems, Minneapolis, MN).

Figure 6. MCF-7 cells secrete both cytoplasmic and nuclear bFGF moieties. Western immunoblot of TCA precipitated 2 M NaCl washes of confluent cells incubated with serum-free media for 48 hours compared with 100 μg of protein from cellular lysates from the MCF-7 constructs.

Figure 7.

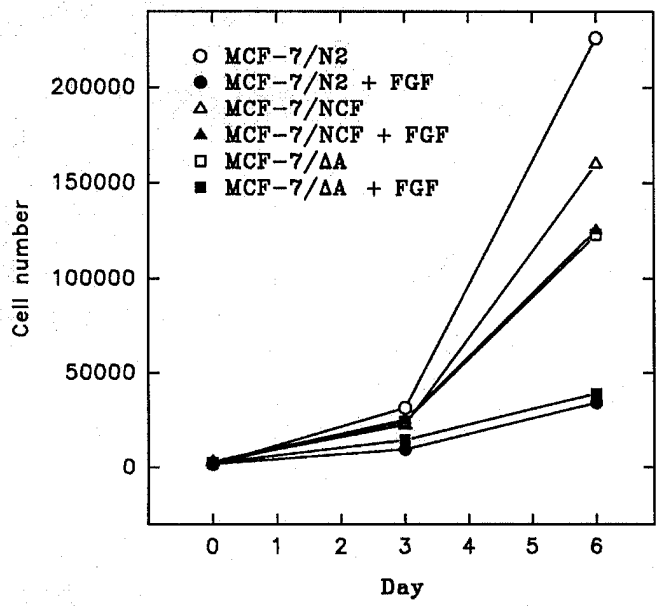

Figure 8.

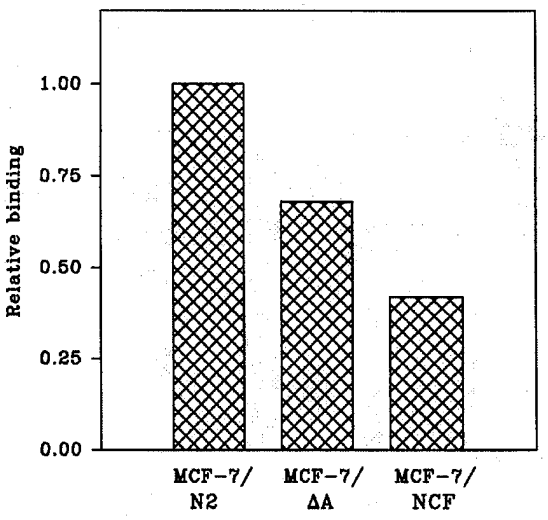

Figure 7. Overexpression of different bFGF moieties in MCF-7 cells modulates growth and response to exogenous bFGF. Cell proliferation kinetics demonstrating growth inhibition by expression of ΔA and NCF in MCF-7 cells compared to N2-transduced controls. N2- and ΔA-transduced cells were inhibited by exogenous 18 kD bFGF but NCF-transduced cells were affected minimally.

Figure 8. Binding of 18 kD bFGF to MCF-7 cells is modulated by endogenously overexpressed bFGF moieties. Relative specific binding of ^{125}I-bFGF to MCF-7/ΔA and MCF-7/NCF cells is presented as a percentage of binding to MCF-7/N2 cells.

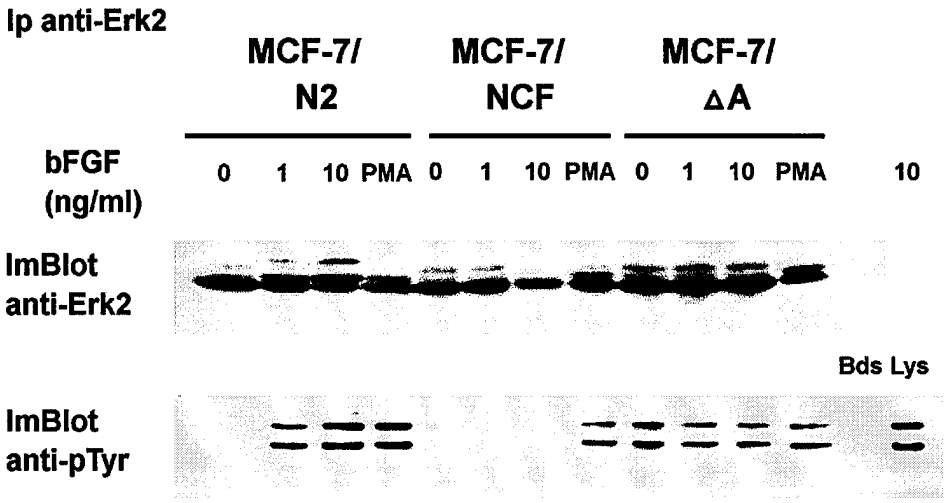

Figure 9. Overexpression of 22 and 24 kD bFGF but not 18 kD in MCF-7 cells inhibits MAP kinase activation by exogenous bFGF but not by PMA. Anti-ERK2 and anti-phosphotyrosine immunostained Western blots of anti-ERK2 immunoprecipitated lysates from MCF-7/N2, MCF-7/ΔA and MCF-7/NCF cells which were treated for 15 minutes with variable concentrations of bFGF or 100 nM PMA.

Expression of the ΔA and NCF vectors in MCF-7 cells inhibited their proliferative capacity compared with N2 controls (figure 7) and arrested the cells in G_1 (not shown). Exogenous bFGF 500 pg/ml decreased proliferation of MCF-7/N2 controls and further decreased the growth rate of MCF-7/ΔA cells, but only had a marginal effect in MCF-7/NCF cells. The data were similar with ^{3}H-thymidine incorporation experiments. To understand the mechanism for lack of response to exogenous bFGF by cells expressing nuclear localizing bFGF species, we undertook binding studies with ^{125}I-labeled 18 kD bFGF. While Scatchard analysis demonstrated a binding constant of 137 ± 112 pM with an estimated number of binding sites of 2606 ± 363 per MCF-7/N2 cell, Scatchard analysis could not be carried out with MCF-7/ΔA or MCF-7/NCF cells due to decreased specific binding. We report in figure 8 a decreased relative specific binding by ^{125}I-labeled 18 kD bFGF in these two cell types, with a greater inhibition in cells secreting nuclear-localizing bFGF moieties. Signal transduction through the mitogen activated pathway was investigated. Whereas MCF-7/N2 cells respond to bFGF by phosphorylating MAP kinase (figure 9), MCF-7/NCF cells do not constitutively phosphorylate MAP kinase, nor can they do so in response to exogenous 18 kD bFGF. However both PMA and insulin (not shown) phosphorylate MAP kinase in MCF-7/NCF cells, suggesting that only FGF receptor-mediated pathways are inhibited. MCF-7/ΔA cells constitutively phosphorylate MAP kinase, and do not incrementally increase the level of phosphorylation in response to exogenous 18 kD bFGF.

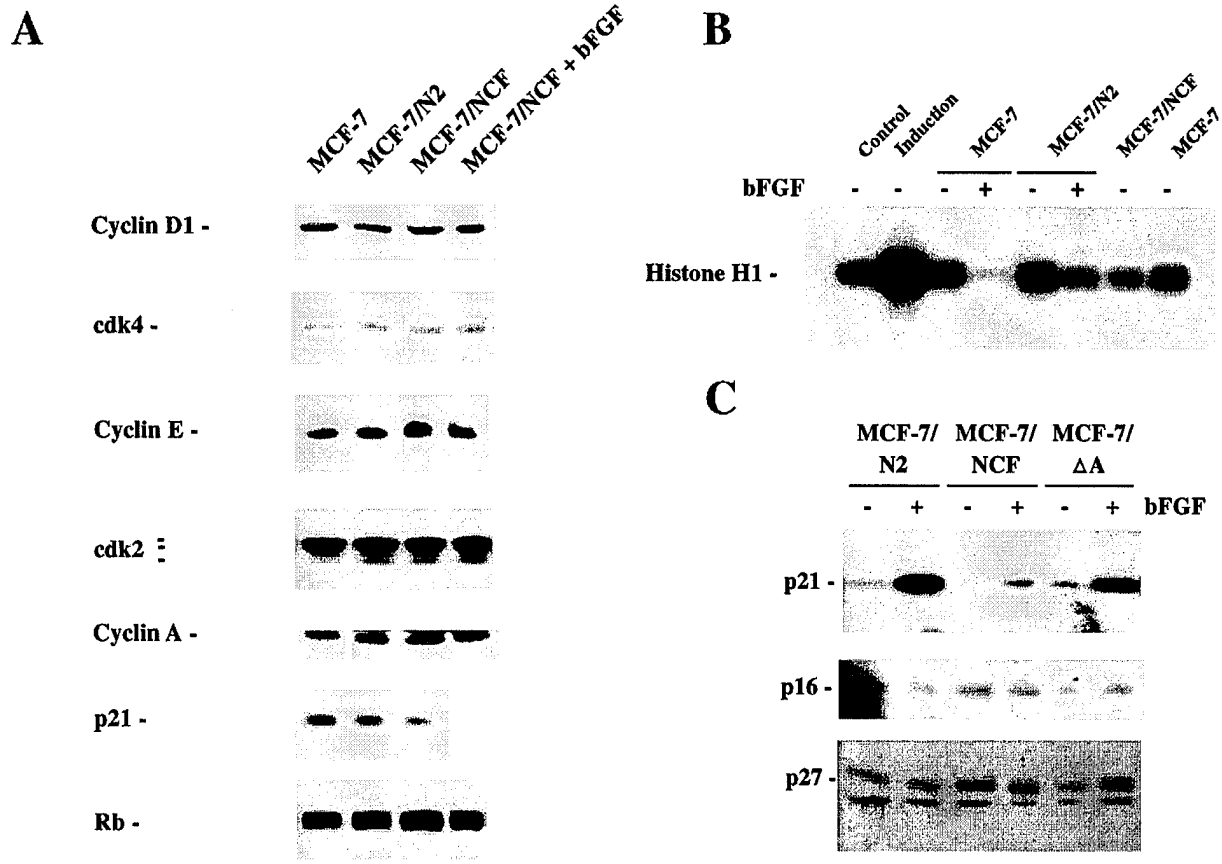

Figure 10. Effects of overexpressing bFGF in MCF-7 cells on G_1 cyclins, cyclin kinases, their inhibitors and cyclin kinase inhibitory activity. A. Western immunoblot of cellular lysates from MCF-7, MCF-7/N2, MCF-7/NCF and MCF-7/NCF cells treated with 10 ng bFGF for 24 hours stained with antibodies to Cyclins D_1, E and A, cdk's 2 and 4, $p21^{WAF1/CIP1}$ and pRb, demonstrating no significant modulation of the levels or active states of these proteins, as described earlier. B In vitro kinase assay of H1 phosphorylation, as described in figure 2, depicting inhibitory activity in lysates of MCF-7 and MCF-7/N2 cells treated with bFGF 10 ng/ml for 24 hours and in MCF-7/NCF cells. C. Western immunoblot of lysates from MCF-7/N2, /ΔA and /NCF cells treated with media or bFGF 10 ng/ml for 24 hours, stained with antibodies to $p21^{WAF1/CIP1}$, $p16^{INK4}$ and $p27^{kip1}$, depicting constitutive upregulation of p27 levels in MCF-7/NCF cells.

Investigating mechanisms of cycle arrest in MCF-7/NCF cells revealed that endogenous expression of cytoplasmic and nuclear-localizing bFGF moieties together had no effect on levels of cyclins D_1, E or A, cdk's 2 or 4, $p21^{WAF1/CIP1}$ or pRb (figure 10A). In addition, cdk2 was not inactivated, and in fact, was probably activated, and Rb was not dephosphorylated. Exogenous 18 kD bFGF had no effect in these cells. The presence of an inhibitor was demonstrated in the in vitro kinase assay described earlier and is shown in figure 10B. Assays for levels of known G_1 cdk inhibitors by Western immunoblot showed that $p21^{WAF1/CIP1}$ and $p16^{INK4}$ levels were not affected constitutively, nor were they modulated by exogenous 18 kD bFGF (figure 10C). The levels of $p27^{kip1}$ were however, elevated constitutively, but did not respond to exogenous 18 kD bFGF. These results were congruous with our previous findings that transforming growth factor (TGF)β levels were elevated in these cells (not shown) and TGFβ modulates expression of $p27^{kip1}$ (83).

Published studies have shown that FGFR4 expressed in cells lacking FGF receptors is incapable of signalling the activation of MAP kinase upon stimulation with FGF (56). We determined the content of the four FGF receptors in cellular lysates and membrane preparations obtained by subcellular fractionation (17) of the FGF-producing and control cells using Western immunoblots with commercial antibodies (figure 11). We demonstrated, in duplicate experiments, a lack of difference in the relative quantity of any of the four receptors among the three cell types. We also showed a decreased amount of FGFR4 associated with the membrane fraction in MCF-7/NCF cells. To complement this finding, we determined the heterodimerization pattern of the four FGF receptors with FGFR1. Figure 12 shows that MCF-7/NCF cells lack tyrosine phosphorylation in one of the four receptors associated with receptor 1 not identifyable from this experiment. The experiment clearly demonstrates, however, that there is a constitutive heterodimerization of FGFR4 with FGFR1 in MCF-7/NCF cells which is not modulated by exogenous 18 kD bFGF. In MCF-7/N2 cells FGFR1 heterodimerizes with all of the other three receptors upon addition of 18 kD bFGF.

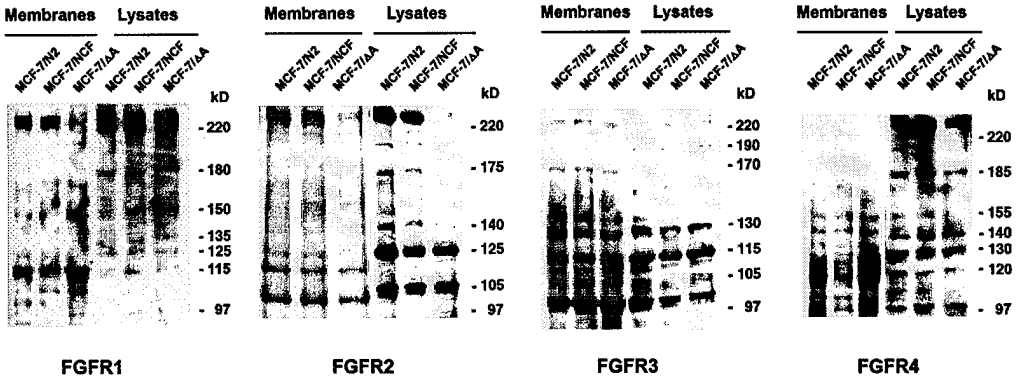

Figure 11. Downmodulation of membrane-associated FGFR4 in cells expressing and secreting both nuclear and cytoplasmic bFGF moieties. Western immunoblots of total cellular lysates from the FGF-expressing and control cells and of TCA precipitated protein from membrane preparations obtained from subcellular fractionation of the same cells.

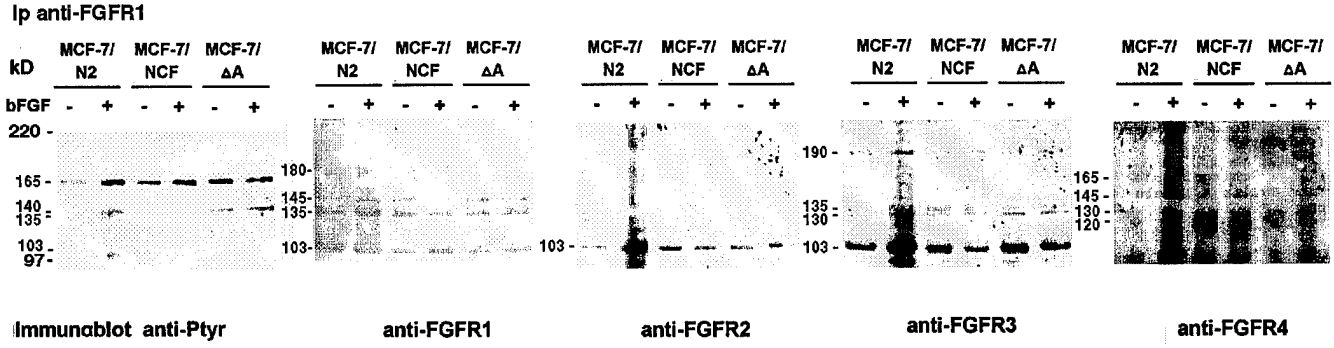

Figure 12. Preferential heterodimerization of FGFR1 with FGFR4 in cells expressing nuclear-localizing bFGF moieties. Western immunoblots of immunoprecipitates of FGF receptors 1-4 from FGF-producing and control MCF-7 cell constructs imunostained with antibodies to phosphotyrosine and FGF receptors 1-4.

Conclusions and significance

These preliminary data support our hypothesis that the 18 kD bFGF moiety activates more than one signal pathway upon binding to high-affinity receptors. One of those pathways is the mitogen activated pathway through MAP kinase, G_1 cyclins D_1 and E and cdk4 causing cycle activation. Another pathway, deviating from the previous one at an unknown point, induces the cell cycle inhibitor $p21^{WAF1/CIP1}$ and induces cdk2 inactivation and Rb dephosphorylation through increased association with cyclin complexes, causing cell cycle arrest in G_1.

Higher molecular weight forms of bFGF probably bind a different set of FGF receptors with different affinities, although such hypotheses are without definitive experimental proof. These moieties do cause preferential heterodimerization of FGFR4 with FGFR1 and internalization of receptor 4. These molecules do not stimulate the MAP kinase stimulatory pathway because FGFR4 acts as a "dominant negative" in the activation of MAP kinase. Consequently, G_1 cyclins are not induced by the mitogenic pathway. $p21^{WAF1/CIP1}$ is also not induced by these moieties, but $p27^{kip1}$ is induced, probably secondary to TGFβ induction. Cells expressing only the 18 kD moiety constitutively phosphorylated MAP kinase to a low level, and were not able to upregulate phosphorylation with addition of exogenous bFGF. This phenomenon was probably due to downregulation of MAP kinase activation, as occurs after 24 hours of exposure by exogenous bFGF (figure 1B). This downmodulation is also probably mediated by TGFβ, at least partially, because antibody to TGFβ increases the 24 hour bFGF induced phosphorylation of $pp42^{mapk}$ (data not shown). Exogenous bFGF does induce increased levels of $p21^{WAF1/CIP1}$ in cells overexpressing 18 kD bFGF, suggesting that the pathway to its induction diverges before MAP kinase in the signal cascade. These conclusions are only possible scenarios which can be deduced from the data, as the high molecular weight moieties were expressed intracellularly, localized to the nucleus and may have used alternate signalling paths not involving receptors. Similarly, the 18 kD moiety expressed intracellularly may use direct cytoplasmic signal pathways not involving cellular receptors.

MCF-7 cells transduced with both cytoplasmic and nuclear bFGF moieties secrete both FGF types. The higher molecular weight bFGF inhibits binding of 18 kD exogenous bFGF to cellular receptors, probably through noncompetitive inhibition, while in cells expressing the 18 kd moiety, inhibition of binding is competitive. These hypotheses will only be proven with competition experiments between the moieties. In addition, these data may also have broader significance, as other, naturally occurring mammary-derived cell lines are also inhibited by bFGF, and their response appears to correlate negatively with their intracellular content of both cytoplasmic and nuclear-localizing bFGF moieties.

A number of manuscripts describing these preliminary data are in preparation and will be submitted for publication in the next several months.

Future work will address the mechanisms through which bFGF mediates signalling towards induction of $p21^{WAF1/CIP1}$, from the four FGF receptors through various divergent signalling paths. Cells lacking FGF receptors will be transfected with each of the four receptors individually and in pairs to determine the receptor combinations transmitting the inhibitory signal. Signal pathways will be interrupted by dominant negative expression vectors for Ras, Raf-1 and MAP kinase to determine the pathway to induction of the inhibitory signal.

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